Chronic Physiological Shear Stress Inhibits Tumor Necrosis Factor–Induced Proinflammatory Responses in Rabbit Aorta Perfused Ex Vivo

Hideyuki Yamawaki, DVM, PhD; Stephanie Lehoux, PhD; Bradford C. Berk, MD, PhD

Background—Regions in the vasculature exposed to steady laminar flow have a lower likelihood for atherosclerosis than regions exposed to disturbed flow with low shear stress. We previously found that laminar flow of short duration inhibited tumor necrosis factor (TNF)-α–mediated proinflammatory signaling in cultured endothelial cells (ECs). However, mechanisms responsible for the atheroprotective effects of physiological shear stress remain undefined. Therefore, we examined the effects of chronic shear stress on TNF-α–induced inflammatory responses using an ex vivo perfusion organ culture system.

Methods and Results—Rabbit aortas were exposed to low or normal shear stress (0.4 or 12 dyne/cm²) at a constant pressure for 24 to 26 hours. EC and vascular smooth muscle cell (VSMC) proteins were selectively purified. After exposure to low shear stress, TNF-α (50 ng/mL, 6 hours) specifically stimulated vascular cell adhesion molecule (VCAM)-1 expression in ECs but not VSMCs. TNF-α–stimulated VCAM expression was inhibited significantly by preexposure to normal shear stress. Normal shear stress inhibited TNF (15 minutes) activation of mitogen-activated protein (MAP) kinases (c-Jun NH₂-terminal kinase [JNK], p38, extracellular signal–regulated kinase [ERK]) in ECs. Specific pharmacological inhibitors of JNK and p38 but not ERK significantly inhibited TNF-induced VCAM expression. Normal shear stress prevented the association of TNF receptor (TNFR)-1 with TNFR-associated factor (TRAF)-2. There was no effect of low or normal shear stress on TNF-α–induced nuclear factor-κB activation. A nitric oxide synthesis inhibitor, N⁵-nitro-L-arginine methyl ester, did not reverse the inhibitory effects of shear stress on VCAM expression.

Conclusions—These results suggest that physiological shear stress is antiinflammatory by specifically inhibiting MAP kinase signaling and inhibiting TRAF-2 interaction with TNFR-1. (Circulation. 2003;108:1619-1625.)

Key Words: blood flow ■ endothelium ■ signal transduction ■ atherosclerosis

It is believed that physiological shear stress exerts atheroprotective effects in vivo, because atherosclerosis occurs preferentially in areas of disturbed flow or low shear stress, whereas regions with steady laminar flow and physiological shear stress are protected. A pathogenic feature of early atherosclerosis is an inflammatory process in which the endothelium is activated by proinflammatory cytokines. In vitro observations that exposure of cultured endothelial cells (ECs) to steady laminar flow prevents expression of inflammatory molecules and monocyte adhesion to ECs support this concept. Recent in vitro studies from our laboratory also showed that flow inhibited mitogen-activated protein (MAP) kinase signaling induced by tumor necrosis factor (TNF)-α. Despite these findings, there are no publications showing that the atheroprotective mechanisms of shear stress on signal transduction found in vitro are physiologically relevant. Previous investigators showed that expression of vascular cell adhesion molecule (VCAM)-1 and monocyte binding were increased in rabbit carotids exposed chronically to low shear stress compared with carotids exposed to normal shear stress. To gain insight into mechanisms responsible for these antiinflammatory effects of shear stress in vivo, we used an ex vivo perfused vessel culture system to study chronic physiological shear stress. The system is optimal for chronic experiments because of better preservation of cell-to-cell interactions, intact extracellular matrix, and maintenance of differentiated cellular phenotypes. Here, we demonstrate the mechanisms by which chronic physiological shear stress exerts antiinflammatory effects on intact vessels.

Methods

Antibodies and Reagents

Antibody sources were as follows: VCAM-1, TNF receptor (TNFR)-associated factor (TRAF)-2, extracellular signal–regulated kinase (ERK)1/2, p38, endothelial nitric oxide synthase (eNOS), actin, 1αB-α, and nuclear factor (NF)-κB p52, Santa Cruz; phospho-ERK1/2, -p38, -eNOS (ser-1177), -Akt (ser-473), c-Jun NH₂-terminal kinase (JNK)2, and Akt, Cell Signaling; phospho-JNK (Promega); TNFR-1, Stressgen; and CD51, DAKO.
Reagent sources were as follows: TNF-α, Roche; JNK inhibitor SP600125, p38 inhibitor SB203580, and ERK inhibitor PD98059, Calbiochem.

Perfusion Organ Culture
Animal experiments were performed according to the guidelines of the National Institutes of Health and American Heart Association for the care and use of laboratory animals and were approved by the University of Rochester Animal Care Committee. Male New Zealand White rabbits (2 to 3 kg; Covance Research Products, Denver, Pa) were anesthetized with ketamine (50 mg/kg IV) and xylazine (2 mg/kg IV). Arterial segments from the descending thoracic aorta were isolated and cannulated at a constant pressure (80 mm Hg).5–11 Isolated aortic segments were connected to a closed perfusion circuit consisting of a 3-port reservoir, a peristaltic pump (Masterflex), and a pressure chamber to control intraluminal hydrostatic pressure. Vessel segments were placed in a bath filled with culture medium identical to that used in the intraluminal compartment, consisting of serum-free DMEM containing antibiotics (100 IU/L penicillin, 100 mg/L streptomycin, and 100 μg/L amphotericin B). Previous reports found that this organ culture method preserved ~100% EC and vascular smooth muscle cells (VSMC) viability.5–11 To obtain a physiological fluid viscosity (0.04 poise), 5% dextran (molecular weight, 70,000: Sigma) was added to the culture medium. Flow rate was adjusted to 7.5 mL/min for low flow and 220 mL/min for normal flow. In conditions of steady laminar flow, shear stress (σ) is determined by flow rate (Q), fluid viscosity (μ), and vessel diameter (d) according to the relationship \( \sigma = \frac{32 \mu Q}{\pi d^2} \). On the basis of this calculation, shear stress was 0.4 dyne/cm² for low flow and 12 dyne/cm² for normal flow. Organ culture of the aortic segments was carried out under sterile conditions in an incubator containing 5% CO₂ at 37.5°C for 24 to 26 hours. Each aorta was divided into 2 segments: one was used as control, and the other was treated with TNF-α (50 ng/mL) in all experiments. There was no contamination by bacteria or lipopolysaccharide, as shown by microscopy of the medium and the absence of VCAM-1 expression basally (see Figure 2).

Purification of EC and VSMC Proteins From Intact Rabbit Aorta
After organ culture, rabbit aortas were washed with cold PBS. To harvest ECs, vessels were opened longitudinally, and 0.2 mL of lysis buffer (20 mmol/L Tris-HCl, pH 8.0, 0.05% Triton X-100, 150 mmol/L NaCl, 2 mmol/L EDTA, 50 mmol/L sodium fluoride, 2 mmol/L sodium orthovanadate, and protease inhibitor cocktail [Sigma]) was applied to the endothelial surface at room temperature for 6 minutes and collected. Next, the adventitia was removed from the aorta. The remaining tissue was harvested for VSMCs by freezing in liquid N₂ and homogenizing with 1 mL of lysis buffer. Protein concentration was determined with the Bradford protein assay (Bio-Rad). By this method, 20 to 30 μg EC protein was obtained from one vessel segment, which was sufficient for several Western blot analyses.

Immunoprecipitation and Immunoblotting
Immunoprecipitation and immunoblotting experiments were performed as described previously.5,7 The resulting autoradiograms were analyzed with NIH Image 1.60. Experiments were performed at least 3 times, and equal loading of protein was ensured by measuring actin expression.

En Face Immunohistochemistry
After organ culture, rabbit aortas were washed with cold PBS, opened longitudinally, fixed with 2% paraformaldehyde for 20 minutes, and then blocked with 10% normal goat serum for 1 hour. Strips were incubated overnight at 4°C with monoclonal anti-CD31 or NF-κB p52 antibody, followed by incubation with fluorescence-conjugated antibody (AlexaFluor, Molecular Probes) for 1 hour. Images of the EC monolayer were observed with a fluorescence microscope (Olympus).

Figure 1. a, ECs and VSMCs can be separated with high purity from rabbit aorta. Specificity was shown by immunoblotting with CD31 or α-SM actin antibody for ECs and VSMCs, respectively. b, En face immunohistochemistry for CD31. Representative images were obtained from aortas, freshly isolated and exposed to low (0.4 dyne/cm²) or normal (12 dyne/cm²) shear stress for 24 hours. c, Representative immunoblots showing expression of CD31 and eNOS in ECs. Protein equal loading was confirmed with total-actin antibody.

Statistical Analysis
Data are shown as mean ± SEM. Statistical evaluation was performed by unpaired Student’s t test, and a value of \( P < 0.05 \) was taken as a significant difference.

Results
Purification of EC and VSMC Proteins and EC Integrity After Organ Culture
To examine the specific effects of TNF-α in ECs and VSMCs, we selectively purified EC and VSMC proteins from intact rabbit aorta. EC or VSMC lysates immunoblotted for the EC marker CD31 or the VSMC marker α-SM actin showed minimal contamination with the other cell type (<5%, Figure 1a), indicative of the successful lysis protocol. We next verified morphology and integrity of ECs after culture. Confluent ECs were uniformly aligned in the direction of flow in freshly isolated aorta (Figure 1b). No morphological change was observed after exposure to low (0.4 dyne/cm²) or normal (12 dyne/cm²) shear stress for 24 hours. Expression of CD31 and eNOS in ECs did not change (Figure 1c). We also confirmed that EC expression of MAP kinases (JNK, p38, ERK1/2), TNFR-1, and TRAF-2 was similar in fresh and organ cultured aorta (data not shown).

Physiological Shear Stress Prevented TNF-α–Mediated EC Activation
To examine the effects of chronic physiological shear stress on the inflammatory response of intact vessels, aortas were preexposed to low (0.4 dyne/cm²) or normal (12 dyne/cm²) shear stress for 20 hours and then stimulated with TNF-α (50 ng/mL) for 6 hours. The inflammatory response was determined by measuring the expression of the adhesion molecule VCAM-1. TNF-α stimulated VCAM-1 expression in ECs after exposure to low shear stress (Figure 2). VCAM-1 expression in ECs was significantly inhibited by exposure to
normal shear stress, by 78±17% (P<0.05, n=3). TNF-α did not induce VCAM-1 in VSMCs.

Physiological Shear Stress Inhibited TNF-α Activation of MAP Kinases in ECs

We next examined MAP kinase activation by TNF-α, which is required for expression of adhesion molecules, including VCAM-1.13–15 TNF-α (50 ng/mL, 15 minutes) stimulated JNK activation in ECs but not in VSMCs after exposure to low shear stress (Figure 3a). Exposure to normal shear stress for 24 hours significantly inhibited TNF-α–stimulated JNK activation by 34±6% (P<0.01, n=3). Of interest, expression of JNK in VSMCs was less than in ECs. Consistent with the JNK results, TNF-α activated p38 (Figure 3b) and ERK1/2 (Figure 3c) in ECs but not in VSMCs after exposure to low shear stress. Similar to JNK, exposure to normal shear stress significantly inhibited TNF-α–stimulated p38 and ERK activation by 38±11% and 36±8%, respectively (P<0.05, n=3). Expression of p38 and ERK in VSMCs was less than in ECs.

JNK and p38 Mediate TNF-α–Induced VCAM-1 Expression in ECs

To evaluate the role of decreased MAP kinase activity in the inhibitory effect of shear stress on VCAM-1 expression, we used specific pharmacological inhibitors. Treatment of rabbit aorta with a JNK inhibitor (30 μmol/L SP600125) or a p38 inhibitor (15 μmol/L SB203580) but not an ERK inhibitor (30 μmol/L PD98059) for 30 minutes significantly inhibited VCAM-1 expression induced by TNF-α (50 ng/mL, 6 hours), by 84±16% and 19±5%, respectively (P<0.01, n=3, Figure 4, a–d). These results suggest that shear stress–mediated inhibition of JNK primarily and of p38 to a lesser extent is responsible for decreased VCAM-1 expression.

Physiological Shear Stress Prevented Association of TNFR-1 With TRAF-2

To determine the mechanism for inhibition of MAP kinase signaling by physiological shear stress, expression of TNFR-1 and TRAF-2 and their interaction were examined. There are 2 receptors for TNF-α, p55 TNFR-1 and p75 TNFR-2. Normally, TNF-α–mediated signaling is mediated primarily by TNFR-1.17,18 The TNF receptor superfamily does not have intrinsic signaling activity, and to transduce signals, TNF receptors recruit adapter proteins called TRAFs. Among 6 TRAF members, TRAF-2 is the primary mediator for TNFR-1 activation of MAP kinase.19 Chronic normal shear stress did not alter expression levels of TNFR-1 and TRAF-2 in either ECs or VSMCs (Figure 5, a and b). However, normal shear stress prevented the association of TNFR-1 with TRAF-2.

Figure 2. Physiological shear stress prevented TNF-α–induced VCAM-1 expression in ECs. After rabbit aortas were preexposed to low (0.4 dyne/cm²) or normal (12 dyne/cm²) shear stress for 20 hours, TNF-α (50 ng/mL, 6 hours) was applied intraluminally. VCAM-1 expression was determined by immunoblotting from 3 independent experiments. Protein equal loading was confirmed with total-actin antibody.

Figure 3. Physiological shear stress inhibited TNF-α–mediated MAP kinases (JNK, p38, and ERK) activation in ECs. After rabbit aortas were preexposed to low or normal shear stress for 24 hours, TNF-α (50 ng/mL, 15 minutes) was applied intraluminally. MAP kinase activation was determined by immunoblotting with phospho-specific antibody. Equal loading was confirmed with total MAP kinase antibodies. Representative blots from 3 independent experiments are shown.
TNFR-1 with TRAF-2 that was observed in ECs exposed to low shear stress (Figure 5c). The present results suggest that a key mechanism for the effects of physiological shear stress is to prevent the interaction of TNFR-1 with TRAF-2. Of note, expression of TRAF-2 in VSMCs was less than in ECs, and 2 major bands for TNFR-1 (~60 and ~46 kDa) were observed. These findings are similar to results obtained by Shubayev and Myers,20 who suggested that the lower band represented cleaved products of TNFR-1.

Shear Stress Did Not Inhibit TNF-α Activation of NF-κB

To show that the inhibitory effects of chronic physiological shear stress on MAP kinase activation are specific, we examined the effect on TNF-α activation of NF-κB, which was shown to regulate VCAM-1 expression.21 To assay this pathway, we first measured degradation of IκB-α, because IkB-α phosphorylation and degradation are required for NF-κB activation and translocation to the nucleus.22 After exposure to low shear stress for 24 hours, TNF-α (50 ng/mL, 15 minutes) stimulated degradation of IκB-α in ECs by 100% (n=3) but not in VSMCs (Figure 6a). Degradation of IκB-α in response to TNF-α was also 100% in ECs after exposure to normal shear stress. We could not detect any clear IκB-α signal after TNF stimulation even in grossly overexposed blots. To further study the effects on NF-κB, we examined translocation of NF-κB from the EC cytoplasm to the nucleus after exposure to both low and normal shear stress (Figure 6b, n=3).

Figure 4. TNF-α–induced VCAM-1 expression was mediated by JNK and p38. After rabbit aortas were incubated in DMEM for 20 hours, MAP kinase inhibitors (SP600125 [SP] 30 μmol/L, SB203580 [SB] 15 μmol/L, and PD98059 [PD] 30 μmol/L) were added for 30 minutes, and then TNF-α (50 ng/mL, 6 hours) was applied. VCAM-1 expression was determined by immunoblotting from 3 independent experiments. MAP kinase activation was determined by use of phospho-specific antibody. Equal loading was confirmed with total MAP kinase antibodies. **P<0.01 from TNF (+).

Figure 5. Physiological shear stress prevents association of TNFR-1 with TRF-2 in ECs. EC and VSMC lysates were obtained after exposure of rabbit aortas to low or normal shear stress for 24 hours. a and b, Expression of TNFR-1 and TRAF-2 were determined by immunoblotting (IB) from 3 independent experiments. c, Interaction of TNFR-1 with TRAF-2 was examined by immunoblotting with TRAF-2 antibody after cell lysates were immunoprecipitated (IP) with TNFR-1 antibody. Equal loading was confirmed with TNFR-1 antibody. Results are representative of pooled samples from 8 rabbits.
Translocation was observed in only a minority of ECs (low shear, 26.0±0.5%; normal shear, 26.0±4.5%). We speculate that the difference in results obtained by immunoblotting compared with immunohistochemistry is a result of the higher sensitivity of immunoblotting than immunohistochemistry and the technical limitations of immunohistochemistry, such as loss of cells during staining and out-of-focus cells. The present results suggest that shear stress inhibition of TNF-α-induced VCAM-1 expression does not involve changes in NF-κB activity.

**Involvement of NO in Shear Stress Inhibition of EC Activation**

Previous studies using cultured ECs suggest that shear stress-stimulated NO production may prevent TNF-α-induced VCAM-1 expression. We found that neither eNOS phosphorylation nor eNOS expression was changed after exposure to low or normal shear stress for 24 hours, with or without TNF stimulation (50 ng/mL, 15 minutes) (Figure 6c, n=3). Consistent with these results, phosphorylation of Akt, an upstream regulator of shear-induced eNOS phosphorylation, also did not change, remaining almost undetectable. Importantly, the eNOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME; 100 μmol/L) did not reverse the inhibitory effects of normal shear stress on VCAM-1 expression (Figure 6d, n=3). This concentration of L-NAME was previously shown to inhibit NO production in the rabbit aorta. These results suggest that NO is not involved in the shear-stress inhibition of VCAM-1 expression by TNF-α.

**Discussion**

The major findings of the present study are that chronic physiological shear stress prevents TNF-α-induced expression of the proinflammatory molecule VCAM-1 in intact vessels. Novel aspects of this study include characterization of these flow-mediated effects in an organ culture system that recapitulates both the pressure and shear stress of the intact vessel and the ability to perform biochemical and signal transduction analysis of ECs and VSMCs specifically. Our results show that the effects of shear stress are specific to ECs and are not observed in VSMCs. A key finding of the present study is that chronic flow at physiological shear stress inhibited TNF-α activation of the MAP kinase pathways, whereas NF-κB was not affected, demonstrating specificity of signal pathway effects. Furthermore, we determined that chronic physiological shear stress prevented TNFR-1 association with TRAF-2, a novel mechanism by which shear stress may exert antiinflammatory effects (Figure 7). These results are the first demonstration of shear stress–mediated inhibition of TNF-α signaling in a chronic model and support our previous findings in cultured ECs, in which we found that steady laminar flow of short duration (10 minutes) inhibited TNF-α activation of JNK. Thus, we show for the first time that the atheroprotective mechanisms of physiological shear stress involve shear stress inhibition of VCAM-1 expression by TNF-α.
stress observed in vitro are physiologically relevant. Our perfusion organ culture system is a powerful experimental tool that bridges the in vivo and in vitro situations.

A key advance required for the biochemical studies reported here was the successful purification of EC and VSMC proteins from intact vessel. Purification of >95% was demonstrated by immunoblotting of specific EC and VSMC markers. To the best of our knowledge, these data represent the first successful purification of EC and VSMC proteins from arterial segments. By this technique, we found that TNF-α selectively stimulated the activation of MAP kinases (JNK, p38, ERK1/2) in ECs but not VSMCs. The lack of response was not because of an inability of TNF-α to interact with VSMCs, because similar results were obtained when TNF-α was added to the inside or the outside of the vessel. The most likely explanation is the low expression levels of TRAF-2 and MAP kinases in VSMCs (Figures 3 and 5). It has been reported that TNF-α stimulates MAP kinases in cultured aortic VSMCs. A likely explanation for this difference is that VSMCs in organ cultured aorta remain in the contractile phenotype and express lower levels of proteins required for TNF signal transduction.

On the basis of the present study and previous work from our group, we believe that inhibition of TNF-α-mediated MAP kinase activation is one mechanism by which flow limits EC inflammatory responses. By performing the inhibitor experiments, we determined that inhibition of JNK primarily mediates the effect of physiological shear stress to decrease VCAM-1 expression. We found that inhibition of individual MAP kinase (~35%) was less than inhibition of VCAM-1 expression (~80%), which is logical, because VCAM-1 gene expression represents a convergence of signal pathways. For example, activation of transcription factor AP-1 is mediated by JNK, p38, and ERK, whereas ATF-2 is mediated by p38 and JNK. Hence, we believe that there are likely to be synergistic effects of inhibiting >1 MAP kinase. Because all MAP kinases were inhibited by shear stress, we hypothesized that a likely mechanism would involve inhibition of proximal signal events in TNFR signaling. We found that physiological shear stress prevented the association of TNFR-1 with TRAF-2 (Figure 5c). Because the binding of TRAF-2 to TNFR-1 is normally induced after TNF stimulation, our data suggest that low shear stress promotes the interaction (and may be associated with production of inflammatory mediators). Similar results were recently reported in VSMCs, in which mechanical stretch increased TRAF-2 binding to TNFR-1 and sensitized the cells to JNK- and p38-mediated apoptosis. Why the NF-κB pathway remains intact after shear stress is unclear. Previous reports support our results, because they showed in TRAF-2-deficient cells that TNF-induced JNK activation was disrupted but NF-κB activation was intact. Another possible mechanism by which shear stress is anti-inflammatory might be via shear stress–stimulated eNOS expression and/or NO production, as previously shown in cultured ECs. However, this is unlikely to be the case in the intact vessel, because we found no difference in eNOS expression in low compared with normal shear stress, and inhibiting NO production with L-NAME did not reverse the inhibitory effects of shear stress on VCAM-1 expression. A recent report by Urbich et al supports our results, because they showed in cultured human umbilical vein ECs that shear stress inhibition of CD40-mediated monocyte chemotactractant protein-1 expression was NO-independent.

In summary, we demonstrate for the first time the atheroprotective benefit of chronic physiological shear stress (12 dyne/cm²) compared with low shear stress (0.4 dyne/cm²) in intact vessels using a perfusion organ culture system. Chronic physiological shear stress prevented TNF-α–mediated VCAM-1 expression in ECs by specifically inhibiting the activation of MAP kinase (mainly JNK and partially p38) but not NF-κB via an NO-independent pathway. A likely mechanism for inhibition of MAP kinases by physiological shear stress appears to be preventing TNFR-1 association with TRAF-2.

Acknowledgments

This study was supported by National Institutes of Health grants HL-62826 and HL-64839 to Dr Berk.

References


Chronic Physiological Shear Stress Inhibits Tumor Necrosis Factor–Induced Proinflammatory Responses in Rabbit Aorta Perfused Ex Vivo

Hideyuki Yamawaki, Stephanie Lehoux and Bradford C. Berk

Circulation. 2003;108:1619-1625; originally published online September 8, 2003;
doi: 10.1161/01.CIR.000089373.49941.C4
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/108/13/1619

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/